ANALYSIS OF ANTIBIOTIC LOADED MATERIALS ON *PSEUDOMONAS AERUGINOSA* AND *STAPHYLOCOCCUS AUREUS* BIOFILM ERADICATION

UNDERGRADUATE RESEARCH THESIS

Presented in partial fulfillment of the requirements for graduation "with honors research distinction" in the undergraduate colleges of The Ohio State University

By

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Abstract

This project investigates the effect of antibiotic loaded calcium sulfate ($CaSO_4$) beads (ALCS-B) and poly(methyl) methacrylate (PMMA) antibiotic infused spacers (both used in treating orthopedic infections) on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm growth and formation in the form of two in vitro studies. The first includes testing the efficacy of various arrangements of ALCS-B through measuring the zone of biofilm inhibition (ZOB-I). The second studies the effect of these beads along with antibiotic loaded PMMA spacers in a "large plate" model, which includes biofilm grown on coupons of various prosthetic materials. When bacterial biofilms are suspected in an infected arthroplasty, antibiotic infused materials such as $CaSO_4$ hemihydrate beads or PMMA spacers are used by some surgeons to achieve the locally high concentrations of antibiotic required to kill the biofilm and avoid further infection. In previous in vitro studies, vancomycin (VAN) and tobramycin (TOB) antibiotic loaded $CaSO_4$ beads demonstrated that sustained antibiotic release could significantly reduce biofilm formation and kill pre-grown biofilms. However, it is unclear whether arrangement of these beads is important for coverage of antibiotics over the infected area. Likewise, a larger model was sought to understand the role of these ALCS-B along with antibiotic infused PMMA spacers.

In the bead spacing model, lawn biofilms of methicillin resistant *S. aureus* SAP231 and *P. aeruginosa* Xen41 were grown on agar for 24 hours, followed by the placement of sixteen VAN+TOB combination antibiotic beads in three different arrangements: 1) all clustered in the center of the plate, 2) arranged circularly towards the edge of the plate, and 3) placed in groups of four equally spaced clusters. Arrangements were compared to a single bead placed centrally. ZOB-I was traced over time using bioluminescent imaging (BLI), and replica plating onto fresh, sterile agar after a period of seven days allowed us to determine whether there were any viable

cells, such as persisters, remaining in the lawn. All sixteen bead arrangements demonstrated significantly more clearing compared to a single bead placement against both pathogens. Further, spaced out sixteen-bead arrangements were able to totally eradicate lawn biofilms, and demonstrated a more rapid killing than arrangements containing beads clustered together, though all three sixteen-bead arrangements eradicated the entire pre-existing Xen41 lawn.

In the large plate model, *P. aeruginosa* Xen4 was grown on coupons of hydroxyapatite, polyethylene, stainless steel 316, and titanium, followed by implementation into a large agar dish containing three antibiotic conditions: 1) an unloaded PMMA spacer (US) containing no antibiotic, 2) a VAN+TOB antibiotic loaded spacer (LS; 2g VAN + 2g TOB), and 3) a VAN+TOB antibiotic-loaded spacer with a 10-cc pack of VAN+TOB antibiotic loaded beads (LS+LB). This model was also visualized using BLI, and colony forming unit (CFU) counts were also performed. The LS+LB condition demonstrated the most biofilm inhibition of the three conditions; moreover, compared to the LS condition, the LS+LB condition produced significantly less bacterial colonies, demonstrating the drastic effect of ALCS-B on biofilm eradication. Together, these studies demonstrate the importance of ALCS-B spacing, as well as the positive implications of merely utilizing loaded beads in reducing biofilms associated with periprosthetic joint infections.

Acknowledgments

The results of the antibiotic-loaded calcium sulfate bead arrangement study have been incorporated into a larger manuscript and recently published in *Materials*, an open access, peer-reviewed journal. The results from the large plate study are currently being integrated into a manuscript and will be submitted to a peer-reviewed scientific journal shortly.

I would like to deeply thank Dr. Paul Stoodley for giving me the opportunity to study in his lab group despite having little prior research experience. Dr. Stoodley assisted me in learning the process of conducting professional and ethical academic research through teaching me various microbiological techniques, educating me about the lab's projects, and allowing me to formulate my own scientific question and experiment for my undergraduate honors research thesis. Dr. Stoodley went over the top in his willingness to assist, teach, and mentor me as an undergraduate researcher for the past two years. I am very appreciative of the time and effort he put into my research experience, and I hope to continue this relationship even after undergraduate graduation. Dr. Stoodley truly embodies the dedication and enthusiasm needed in his work, and I was grateful to have the opportunity to grow under his tutelage throughout my undergraduate career. I hope other students have the ability to study and work under his guidance in the future.

I would also like to thank Nicholas Farrar for mentoring me throughout my first year in the lab and helping me learn valuable scientific techniques and approaches. Nick laid the groundwork for my laboratory experience and was always keen to assist my learning in any way possible. Further, I would like to thank Devendra Dusane, Casey Peters, and Kelly Moore for allowing me to become involved in numerous projects and for assisting with some of the imaging and CFU counts completed in this study. Specifically, I would like to acknowledge the

assistance of Casey Peters in constructing the ALCS-B arrangement models shown in Figure 2. Additionally, I would like to thank Devendra Dusane for his part in helping construct Figures 12, 13, S1, and S2. Lastly, I would once again like to thank Dr. Paul Stoodley for aiding in the organization of Figure 10.

I would like to also acknowledge the substantial and generous support of Biocomposites Ltd., who provided all of the Stimulan materials for my experiments and contributed other experimental design ideas to make my project possible.

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Poster Presentations

1. Dusane, D.H., **Brooks, J.**, Sindeldecker, D., Peters, C.W., Delury, C., Aiken, S.S., Laycock, P., Sullivan, A., Granger, J.F., Stoodley, P. Killing of persister cells and biofilms of *Pseudomonas aeruginosa* by spatial distribution of antibiotic-loaded calcium sulfate beads. Musculoskeletal Infection Society (MSIS) **29th Annual Open Scientific Meeting. New York City, NY.** Aug 02 - 03, 2019.
2. **Brooks, J.**, Farrar, N., Dusane, D.H., Granger, J., Sullivan, A., and Stoodley, P. Analysis of Calcium Sulfate Antibiotic Bead Arrangement on Methicillin Resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* Biofilm Eradication. **18th Annual College of Medicine Trainee Research Day, Columbus, OH,** April 11, 2019.
3. Farrar, N., **Brooks, J.**, Li, A., Dusane, D.H., and Stoodley, P. Antibiotic Resistance and Variant Colony Production of MRSA Biofilms on Prosthetic Joints. **18th Annual College of Medicine Trainee Research Day, Columbus, OH,** April 11, 2019.
4. **Brooks, J.**, Farrar, N., Dusane, D.H., Granger, J., Sullivan, A., and Stoodley, P. Analysis of Calcium Sulfate Antibiotic Bead Arrangement on Methicillin Resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* Biofilm Eradication. **25th Annual Denman Research Forum, Columbus, OH,** February 20, 2019.
5. Dusane, D.H., **Brooks, J.**, Laycock, P., Aiken, S., McPherson, E., Sullivan, A., Granger, J.G and Stoodley, P. Antibiotic loaded bead spacing is important in controlling Pseudomonas aeruginosa and Staphylococcus aureus biofilms in periprosthetic infections: an in vitro study. **8th ASM conference on Biofilms, Washington, DC,** October 7-11, 2018.
6. **Brooks, J.**, Farrar, N., Dusane, D.H., Granger, J., and Stoodley, P. Analysis of Antibiotic Tolerant Colonies in Methicillin Resistant *Staphylococcus aureus* Biofilm after Long-Term Antibiotic Treatment. **24th Annual Denman Research Forum, Columbus, OH,** April 3, 2018.

Publications

1. Dusane, D.H., **Brooks, J.R.**, Sindeldecker, D., Peters, C.W., Li, A., Farrar, N.R., Diamond, S.M., Knecht, C.S., Plaut, R.D., Delury, C., Aiken, S.S., Laycock, P.A., Sullivan, A., Granger, J.F., Stoodley, P. (2019). Complete Killing of Agar Lawn Biofilms by Systematic Spacing of Antibiotic-Loaded Calcium Sulfate Beads. *Materials*, *12*(24), 4052.

Fields of Study

Major Fields: Biochemistry, Pre-Medicine

Minor Fields: Bioethics, Economics

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1. Introduction

Bacterial infection acts as a serious obstacle that can result from total joint arthroplasty and other orthopedic surgical procedures. Periprosthetic joint infections (PJI) are reported in 1–1.4% of all total joint arthroplasties[1], and these infections are often difficult to treat, sometimes leading to chronic conditions requiring prosthesis removal. This can result in morbidity, including loss of function and amputation, or mortality[2].

The principal cause of many of these surgical site infections is thought to be bacterial biofilms. Biofilms include thin aggregates of bacteria that adhere to either living or non-living physical surfaces and exhibit increased antibiotic tolerance compared to their planktonic counterparts[3,4]. Evidence indicates that the microorganisms within biofilms can resist antibiotic action, even at high concentrations, which results in the need for sustained, localized, and systematic antibiotic delivery when treating these infections clinically[5,6]. Bacteria convert from their planktonic form to produce bacterial communities when adhered to a material sufficient for organismal growth and division[7]. Ultimately, a biofilm is formed that becomes increasingly tolerant to antibiotic action, even without gaining concrete antibiotic resistance characteristics[8].

Pseudomonas aeruginosa (PA) and *Staphylococcus aureus* (SA) are two common pathogens associated with periprosthetic joint infection[3]. *P. aeruginosa* is a gram-negative, easily adaptable bacterium, while *S. aureus* is a gram-positive microbe that is a leading cause of many infections in the human body, including bone and joint infections[9]. Both pathogens have excelled in enduring and adjusting to the challenge of antibiotic by using an assortment of approaches; namely, through developing antibiotic-tolerant biofilms[10].

In attempt to prevent further infection to an already revised arthroplasty, antibiotic infused materials such as poly(methyl) methacrylate (PMMA) spacers or calcium sulfate

($CaSO_4$) hemihydrate beads are often placed at the site of infection by surgeons in hopes of lowering the infection and eradicating the biofilm[11]. While PMMA is commonly used in a structural manner for implant stabilization[13], $CaSO_4$ aids in the regeneration of bone and is not frequently used on a structural basis. Stimulan is an engineered, naturally absorbable, and mineral-based $CaSO_4$ that has been approved for placement at the site of infection as a void filler. Though not as mechanically robust as PMMA, $CaSO_4$ beads may offer benefits in the elution of antibiotic in order to better treat infections[14]. Nevertheless, both PMMA spacers and $CaSO_4$ beads are commonly infused with antibiotics such as Vancomycin (VAN) and Tobramycin (TOB), which work to suppress bacteria by inhibiting the bacterial cell wall and binding to bacterial ribosomal subunits (preventing protein synthesis), respectively. PA and SA have been shown to be somewhat susceptible to these antibiotics in previous in vitro studies[11], and a combination of the two are many times used when the specific bacteria is unknown.

1.1 Aim of Studies

Though surgeons are sometimes able to treat biofilm infection with antibiotics in these materials, there is little known about the most efficacious manner to use these resources. Specifically, the importance of positioning these antibiotic loaded calcium sulfate beads (ALCS-B) was studied to better determine the need for spacing these materials in the wound when treating PJI's. In the past, we had studied the killing of *P. aeruginosa* Xen41 and *S. aureus* SAP231 bioluminescent lawn biofilm by a single VAN+TOB combination antibiotic loaded $CaSO_4$ bead[12]. In this study, various arrangements of sixteen combination VAN+TOB ALCS-B were analyzed against these pathogens to better understand the importance of spacing in eradicating an in vitro lawn biofilm. Moreover, this study also sought to determine if biofilm could be totally eradicated from antibiotic tolerant phenotypes using these ALCS-B in different arrangements.

Additionally, to obtain a more comprehensive idea of the impact that these beads have on biofilms, a large plate model was designed in order to implement antibiotic against *P. aeruginosa* Xen41 in the form of both PMMA bone cement spacers and a larger amount of ALCS-B. By testing a 10-cc pack of these beads, or the same amount used when employed by surgeons, a better application to a clinical scenario could be analyzed to juxtapose the effect of ALCS-B and the influence of antibiotic infused PMMA spacers. This study also allowed for the formation of biofilms on four different types of coupons, used to simulate biofilm adhering to materials found in prosthetics. The objective of the large plate study was to clarify the effectiveness of ALCS-B while determining the possibility of eradicating a PA biofilm that is more pertinent to a clinical scenario.

1.2 Hypotheses

In the ALCS-B spacing study, it was thought that ALCS-B arrangements that avoid clustering in one area would be more efficacious in eradicating both PA and SA lawn biofilm, along with other antibiotic tolerant bacterial cells. Thus, it was expected that arrangements containing the most equally spaced ALCS-B would provide the most efficient total eradication of biofilm. In addition, it was posed that sixteen ALCS-B instead of one, and accordingly, more concentration of antibiotic, would produce total biofilm killing.

In the large plate model, it was hypothesized that the condition containing an antibiotic loaded PMMA spacer and antibiotic loaded $CaSO_4$ beads (LS+LB) would produce the most inhibition of PA biofilms grown on various coupons. However, due to the large concentration of antibiotic in a loaded spacer, it was expected that the loaded PMMA spacer only condition (LS) would not differ significantly from the LS+LB condition. It was proposed that both the LS and LS+LB conditions would outperform the US control condition in terms of biofilm killing.

2. Materials and Methods

2.1 Preparation of Stimulan Beads and Simplex Spacers

2.1.1 Preparation of Stimulan® Antibiotic Loaded Calcium Sulfate Beads (ALCS-B)

All experiments performed required the manufacturing of Stimulan (Biocomposites, Ltd., Staffordshire, England) beads with antibiotic infusion. ALCS-B were prepared using Stimulan® Rapid Cure 10-cc mixing kits to mimic clinical antibiotic treatment of biofilm infection. 20 g of Stimulan $CaSO_4$ powder was mixed with 1000 mg of Vancomycin Hydrochloride (VAN) and 240 mg of Tobramycin Sulfate (TOB) antibiotics (Gold Biotechnology Inc., MO, USA) according to the instructions for use of Stimulan[15]. Once blended, the 6 mL of sterile solution included in the kit was added, and the mixture was stirred into a uniform paste for 30 seconds. The paste was pressed into a flexible mold (Biocomposites, LTD., Keele, UK) with bead spaces of 4.8 mm in diameter. The hemispherical beads were then removed from the mold after a setting for 10-15 minutes at 20 °C. The preparation procedure is shown in Figure 1.

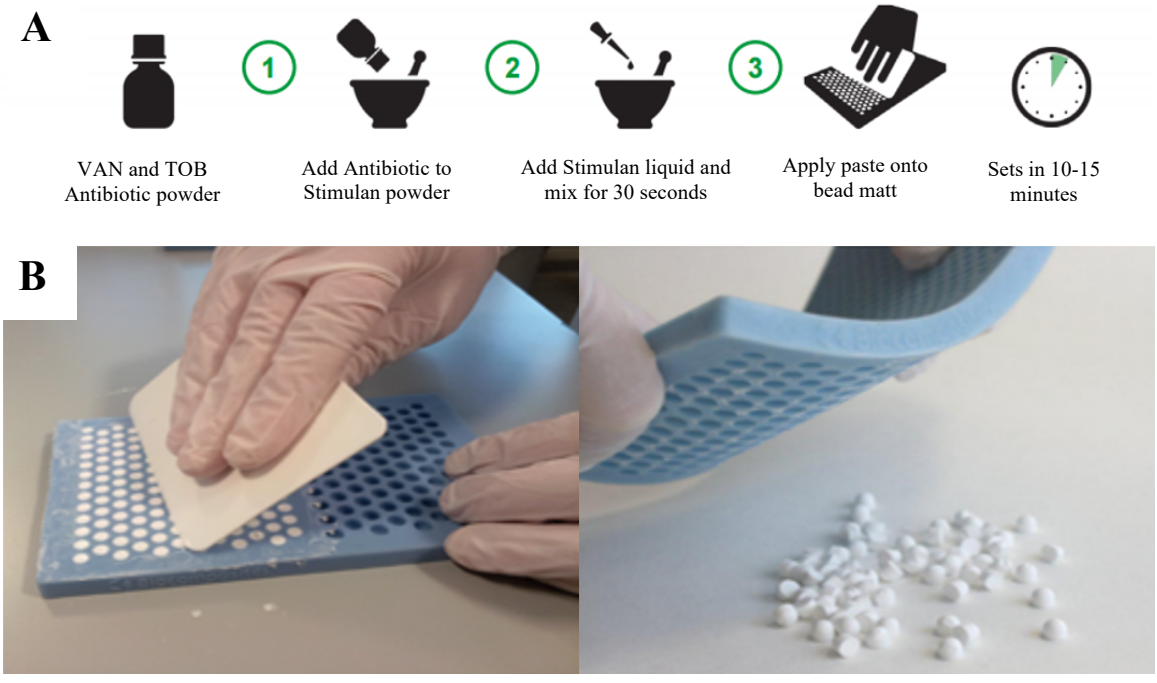

Figure 1A-B. Step-by-step protocol for mixing Stimulan beads with VAN+TOB antibiotics (A) and images of spreading the Stimulan beads on the bead matt and removing the beads once hardened[16] (B). One 10-cc Stimulan pack contains 20 g $CaSO_4$ powder and 6 mL of liquid.

2.1.2 Preparation of Simplex™ PMMA Antibiotic Loaded Spacers

Experiments conducted with the large plate model required construction of Simplex™ PMMA spacers (Stryker® Howmedica Osteonics, NJ, USA) either with or without antibiotic infused. Simplex™ P SpeedSet™ Radiopaque Bone Cement construction kits along with large circular molds were used to prepare spacers in the shape of a 3.45 cm diameter thick disc. When fabricating VAN+TOB antibiotic infused PMMA spacers, 40 g of Simplex™ PMMA bone cement powder was combined with 2 g VAN, 2 g TOB, and mixed similarly to the Stimulan procedure above. Unloaded spacers were prepared with only Simplex™ PMMA bone cement powder and no antibiotic. 20 mL of sterile Methyl methacrylate liquid was then added to the mixture, stirred into a dough-like mass for 1-2 minutes, and then poured into the 3.45 cm diameter circular mold to set for 30 minutes at 20 ˚C[17].

2.2 Bacteria and Growth Conditions

Bioluminescent, bioengineered strains of *Pseudomonas aeruginosa* PAO1 derivative (Xen 41, PerkinElmer, MA, USA) and *Staphylococcus aureus* (SAP231, an altered USA 300 MRSA strain) were used in this study. *P. aeruginosa* Xen 41 was grown in Tryptic Soy Broth (TSB; Becton, Dickinson & Company, MD, USA) while *S. aureus* SAP231 was developed in Brain Heart Infusion broth (BHI; Becton, Dickinson & Company). Both liquid cultures were cultivated overnight at 37 °C with shaker conditions set to 200 RPM.

2.3 Bioluminescent Imaging (BLI)

Every 24 hours, BLI was implemented using an *in vitro* imaging system (IVIS 100, Xenogen, MA, USA) on each individual petri dish and each large plate. BLI measures the relative amount of actively growing biofilm. Images were captured in both grayscale intensity, where white suggested the most metabolically active and black demonstrated no activity, as well as in color, where red represented the highest activity and blue or black exemplified no activity. No bioluminescent activity in images could be qualified as removal of biofilm, inactivation, killing, or a combination of the three. White light (plain) images of each plate were also captured with a digital camera on a cell phone. For BLI of the large plate, each quadrant of the plate was imaged due to inability to capture the whole plate in one image. The quadrant images were then stitched together using Photoshop (Adobe).

2.4 Antibiotic Loaded Calcium Sulfate Bead (ALCS-B) Spacing Model

2.4.1 Biofilm Formation on Solid Agar

For the bead spacing study, broth cultures of *P. aeruginosa* Xen41 and *S. aureus* SAP231 were diluted to 1% after 24 hours and used as an inoculum for biofilm formation on sterile petri dishes containing Tryptic Soy Agar (TSA; Sigma-Aldrich) and Brain Heart Infusion agar (BHI; Sigma-Aldrich), respectively. 100 μL of culture was added to petri dishes of 85.5 mm diameter (Fisher Scientific, ON, CAN) containing 24 mL of the necessary agar, and spread among the plate. The plates were then incubated at 37 °C for 24 hours to establish a lawn biofilm.

2.4.2 Antibiotic Loaded $CaSO_4$ Bead Arrangement

In attempt to eradicate a pre-existing biofilm, sixteen Stimulan VAN+TOB ALCS-B were pressed into the agar of the 24-hour lawn biofilms in three different arrangements (Figure

2B-D). These sixteen bead arrangements were compared to a single VAN+TOB bead placed centrally (Figure 2A). ALCS-B were placed onto the lawn after a 24-hour period of incubation to mimic antibiotic treatment of an existing infection. Plates were then incubated at 37 °C for a period of 7 days. Plates were imaged every 24 hours using both BLI and white light (plain) imaging to track the suppression of metabolic activity.

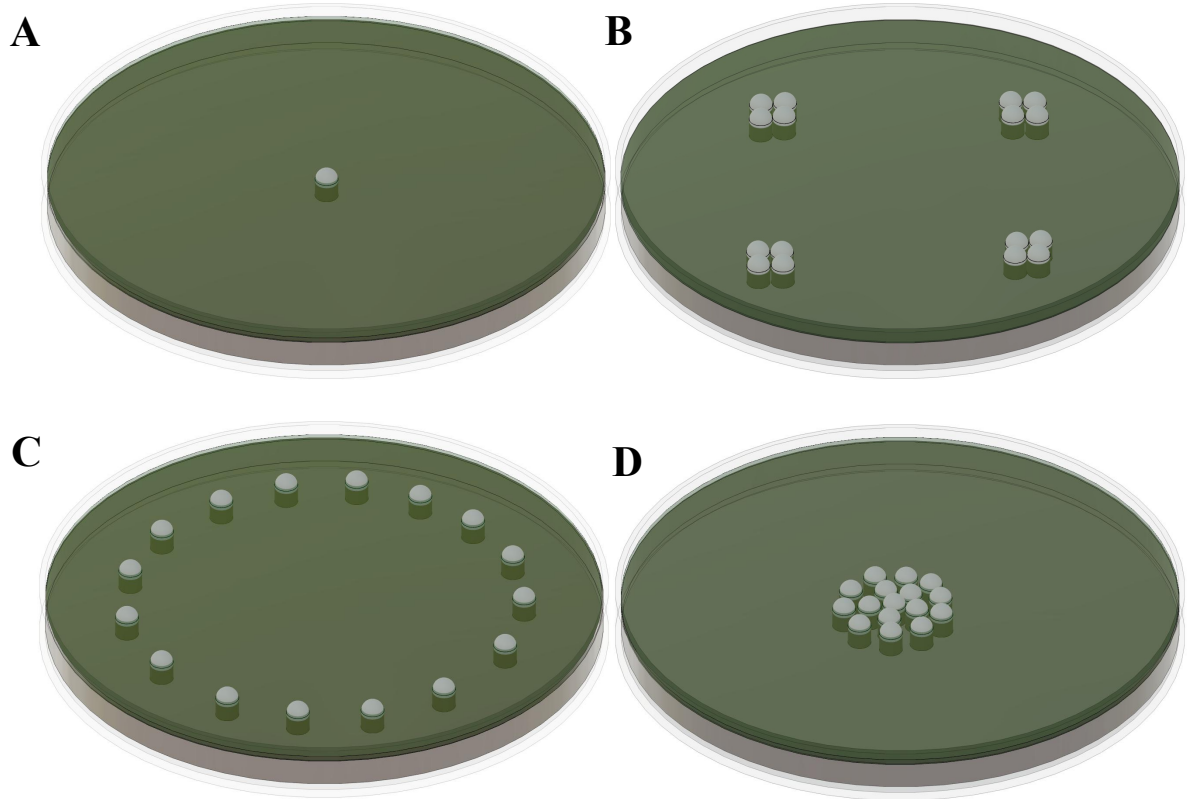

Figure 2A-D. Models of the various VAN+TOB ALCS-B arrangements used against PA Xen 41 & SA SAP231 existing biofilms after incubated for 24hr. The arrangements include a single bead placed centrally (A) and sixteen beads in the following arrangements: placed in groups of four equally spaced clusters (B), arranged circularly towards the edge of the plate (C), and all clustered in the center of the plate (D).

2.4.3 Replica Plating

After an incubation period of 7 days, plates from each arrangement type were chosen to be replica plated. This was completed to determine if biofilm was completely eradicated by

antibiotic treatment or solely growth inhibited. Original plates were neutralized by removal of ALCS-B, and then pressed face down on a replica plating apparatus draped with sterile velveteen cloth (Figure 3). The original plate was then removed, and a fresh petri dish containing only growth medium (TSA or BHI agar) was pressed onto the cloth to transfer genetic material. Replica plates were then incubated at 37 °C for a period of 5 days and imaged every 24 hours using BLI and white light (plain) imaging.

Figure 3. Replica plating apparatus containing velveteen cloth on top of the replica plating base[18]. This was used to transfer genetic material to a fresh agar plate after VAN+TOB ALCS-B arrangements had been exposed to PA or SA biofilm for 7 days.

2.4.4 Statistical Analysis of Zone of Biofilm Inhibition (ZOB-I)

The zone of biofilm inhibition (ZOB-I), between different bead arrangements was quantified (in mm^2) at each time point throughout the experiment using ImageJ image analysis software along with the IVIS images. Statistical comparisons between arrangements were implemented through Excel (Microsoft) software using an unpaired two-tailed, Student's t-test assuming equal variances. Differences with $p<0.05$ were deemed significant. Original ALCS-B arrangement experiments were performed in triplicates, compared to replica plating, which was completed in duplicates.

2.5 Large Plate Model

2.5.1 Biofilm Formation on Coupons

For the large plate study, broth overnight cultures of *P. aeruginosa* Xen41 were diluted to 0.1% after 24 hours and used as an inoculum for biofilm formation on sterile coupons of four different materials: Hydroxyapatite (HA), Stainless Steel (SS-316), Polyethylene (PE), and Titanium (Ti). 4 mL of 0.1% diluted overnight culture was added to four different wells of a six-well plate, and three coupons of each material were submerged in the inoculum. The six-well plate was incubated at 37 ˚C for 72 hours to establish 3-day biofilms adhered to these coupons.

2.5.2 Building the Large Plate Model

To build the large plate model, 50 mL of TSA agar was added to a large pie dish and allowed to solidify, followed by placement of a PMMA spacer (either loaded with antibiotic or unloaded) along with another 100 mL aliquot of molten agar. Once hardened, three of each coupon material containing a 3-day biofilm were placed around the spacer according to Figure 4. A 10-cc pack of ALCS-B was then spread throughout the plate if necessary, with beads placed in heavy amounts near the spacer and sparse amounts near the edges. In two of the three conditions (described in section 2.5.3), no beads were added. Finally, another 100 mL of hot agar was poured to cover the coupons and beads. The apparatus was allowed to cool and then covered with Saran wrap for imaging and incubation.

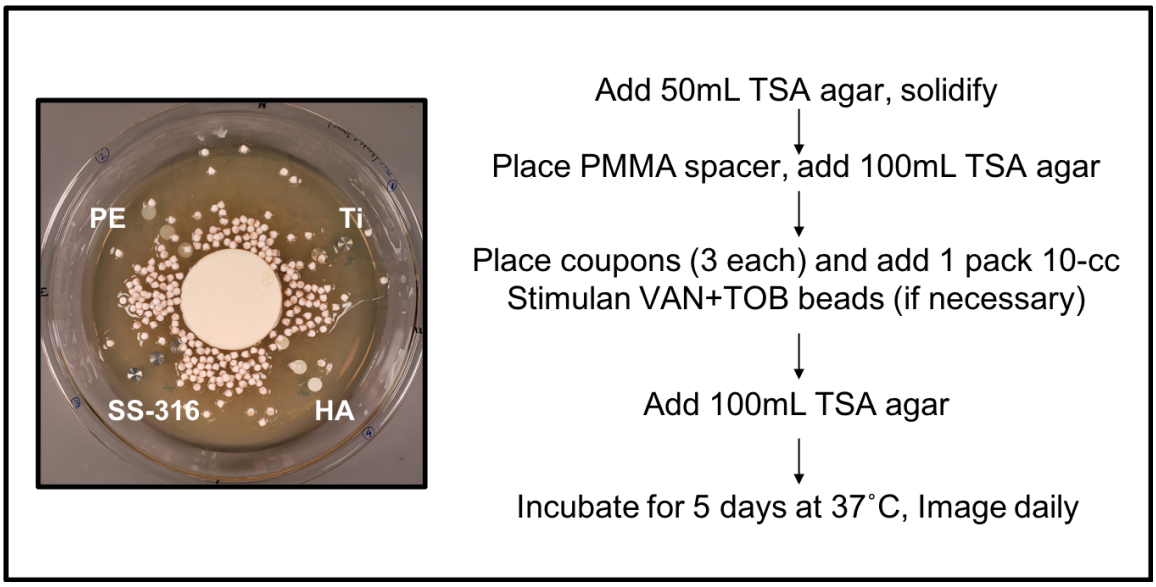

Figure 4. Fully assembled large plate (directly after set-up) along with step-by-step set-up instructions of the model. Three coupons of each material were placed in a straight line moving away from the spacer. The coupon types are labeled on the image of the large plate.

2.5.3 Antibiotic Conditions

To examine the role of antibiotic infused PMMA spacers compared to ALCS-B, three antibiotic conditions of the large plate were tested. The first condition contained an unloaded Simplex™ PMMA spacer only. containing no antibiotic (US). The next condition contained a VAN+TOB antibiotic loaded spacer only (LS), and the final condition contained a VAN+TOB loaded spacer with a 10-cc pack of Stimulan VAN+TOB ALCS-B (LS+LB). The large plates were incubated at 37 ˚C for 5 days and imaged every 24 hours using BLI and a phone camera.

2.5.4 Colony Forming Unit (CFU) Counts

CFU's were performed on all large plates after five days of incubation using an agar plug method. To determine CFU counts, 1.35 cm glass plugs were used to punch out samples containing agar and the respective coupon, followed by placement in 15-mL Falcon tubes. 10 mL of phosphate-buffered saline (PBS) was added to the tubes and then the samples were vortexed. A 10-fold serial dilution with PBS was performed (total well volume of 200 µL) for each sample,

and 10 µL of each dilution was spotted on a TSA agar plate. The spotted plates were incubated upright at 37 ˚C for 24 hours, and colonies were enumerated to determine CFU's in terms of CFU/mL per area of the glass plug in cm^2. CFU counts of each antibiotic condition were compared to determine relative number of viable bacteria after antibiotic treatment.

To quantify the initial number of bacteria on coupons, CFU's were performed after coupons had been incubated for 3 days in the TSB broth inoculum. These coupons were rinsed with PBS and then vortexed in 10 mL of PBS, continuing the protocol mentioned above, since no agar plug could be taken of the liquid media. These counts were reported in terms of calculated coupon area as log CFU/mL per cm^2.

2.5.5 Statistical Analysis of the Large Plate Model

The number of colony forming units was quantified in terms of $\log_{10}$CFU/mL/cm^2 for each coupon material. Coupons of the same material were labeled #1 through #3, where coupon #1 denoted the coupon closest to the spacer, and coupon #3 denoted the coupon furthest from the spacer. Original large plate experiments and CFU counts were both conducted in duplicate. Statistical comparisons between arrangements were implemented through Excel (Microsoft) software using an unpaired two-tailed, Student's t-test assuming equal variances, with $p<0.05$ deeming a significant difference between samples.

3. Results

3.1 ALCS-B Spacing Experiment

3.1.1 *In Vitro* Imaging System (IVIS) Image Analysis

Petri dishes of PA Xen41 and SA SAP231 lawn biofilm with various arrangements of ALCS-B were imaged every 24 hours for 7 days using bioluminescence imaging (BLI) and white light (plain) imaging. Figures 5 and 6 show the area of inhibited bacteria over time for PA Xen41 and SA SAP231 respectively. As stated in section 2.3, a red color denotes the highest bacterial activity while the black color suggests the inactivity of bacterial lawn. All arrangements produced unique zones of biofilm inhibition (ZOB-I) based on the specific arrangement of beads. For instance, a single bead placed centrally produced a typical circular ZOB-I, while the four clusters of four bead arrangement produced four separate circular zones that eventually interact. Compared to one ALCS-B placed centrally, which did not fully inhibit the lawn by day 7, all sixteen bead arrangements seemed to fully suppress the PA Xen41 biofilm by day 4, but not until day 6 in SA SAP231. Moreover, in the single bead placement, antibiotic tolerant cells began to surface on day 4 in the PA Xen41 lawn and can be observed clearly on day 5 (Figure 5). Antibiotic tolerant phenotypes were also confirmed under white light for this single VAN+TOB bead against *P. aeruginosa*. No antibiotic tolerant phenotypes were observed in any sixteen-bead arrangements for both pathogens and the single bead placement against *S. aureus* SAP231.

In comparison of the different sixteen-bead arrangements against PA Xen41, the arrangement of four equally spaced clusters and circularly placed beads seemed to demonstrate a more suppression of metabolic activity in the first two days, though all sixteen bead arrangements demonstrated total bacterial suppression after day 3 (Figure 5). Against SA SAP231, a similar trend was suggested since both the four clusters of four bead and the circular

bead arrangements suppressed all metabolic activity by day 5, compared to the centrally clustered sixteen-bead arrangement, which produced bioluminescence until day 6 (Figure 6). Nevertheless, only the single bead arrangement demonstrated SA SAP231 bioluminescence on day 7.

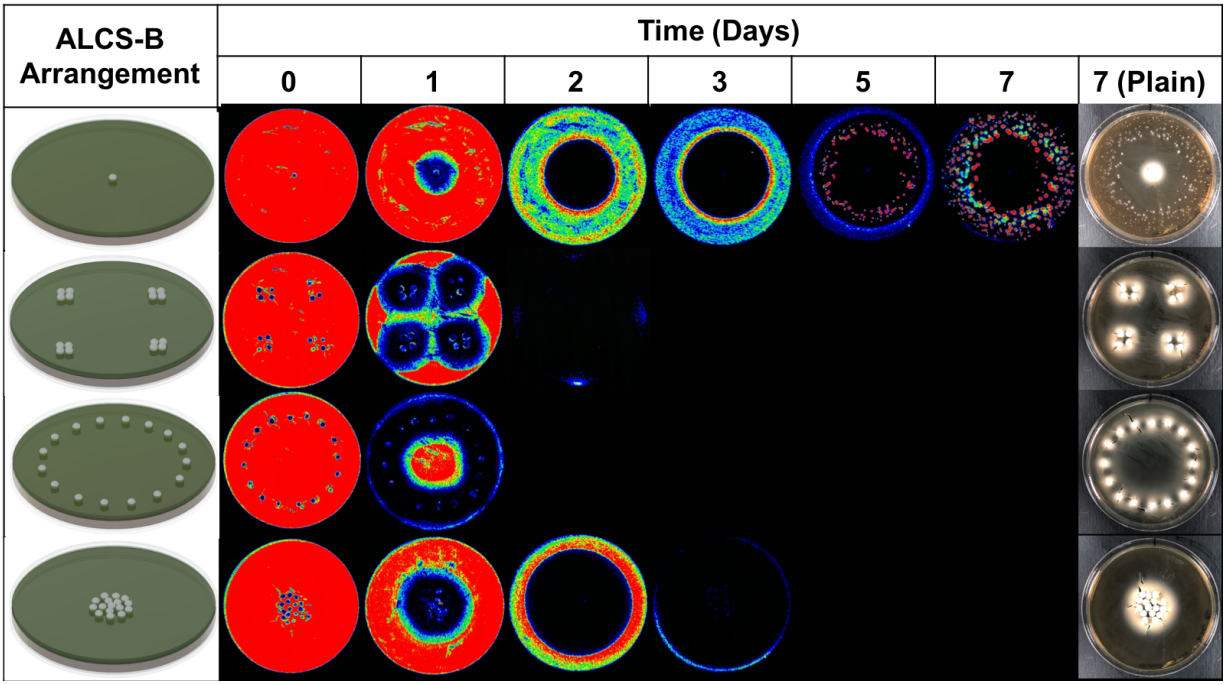

Figure 5. IVIS images of various VAN+TOB ALCS-B arrangements, showing the suppression of *P. aeruginosa* Xen41 lawn biofilms over a period of 7 days. Plain images of each plate are depicted on day 7.

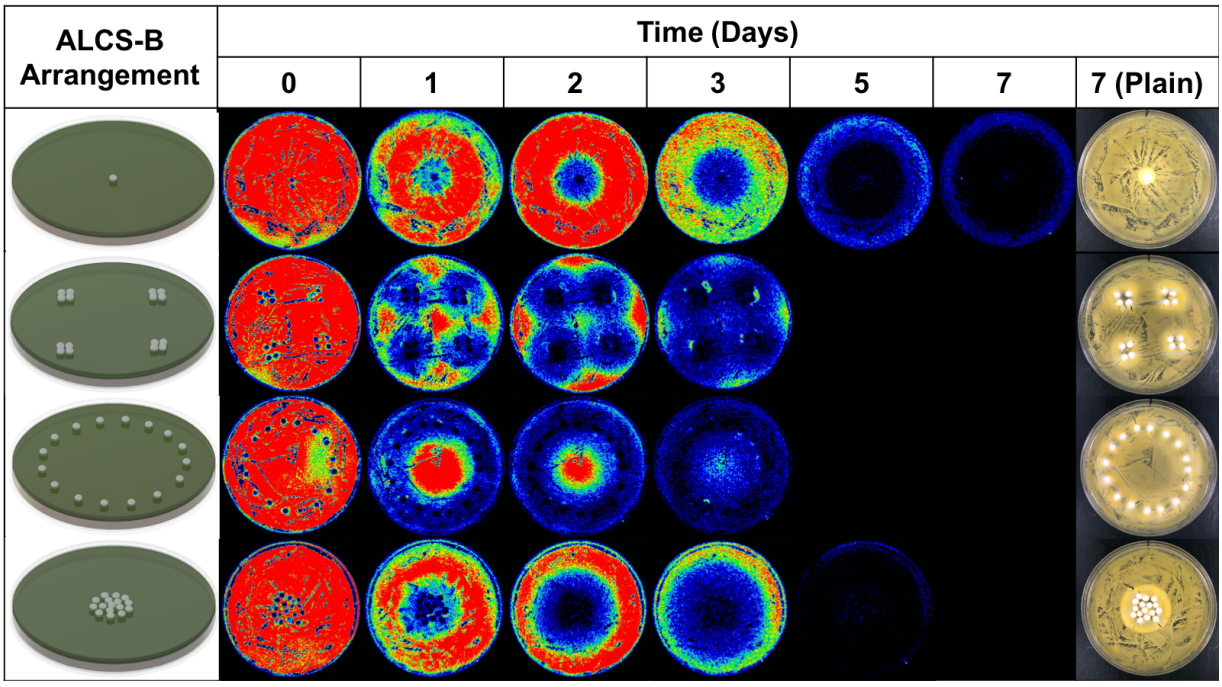

Figure 6. IVIS images of various VAN+TOB ALCS-B arrangements, showing the suppression of *S. aureus* SAP231 lawn biofilms over a period of 7 days. Plain images of each plate are depicted on day 7.

3.1.2 Replica Plating

After a period of 7 days, replica plating was conducted in order to determine if the bacteria suppressed after 7 days was truly eradicated. By transferring the genetic material onto a fresh agar plate, the presence or lack of bacterial growth was analyzed to conclude biofilm eradication versus mere bacterial inhibition by antibiotic. Growth was also examined to determine the presence of antibiotic tolerant phenotypes appearing from the inhibited lawn.

For PA Xen41 replica plates (Figure 7A-B), IVIS and plain images showed no bacterial growth on each of the 5 days for all sixteen-bead arrangements; however, the single ALCS-B arrangement generated bacterial growth along the edges of the plate after 1 day of incubation. For replica plates of SA SAP231 bead arrangements (Figure 8A-B), the four clusters of four and circular sixteen-bead arrangements produced no bacterial growth over 5 days, similarly to these bead arrangements in PA Xen41. Yet, the centrally clustered sixteen-bead arrangement in

SAP231 did generate bacterial growth on both IVIS and white light imaging after 1 day, which was different from its PA Xen41 counterpart. The single bead placement also exhibited bacterial growth on the replica plate after 1 day of incubation.

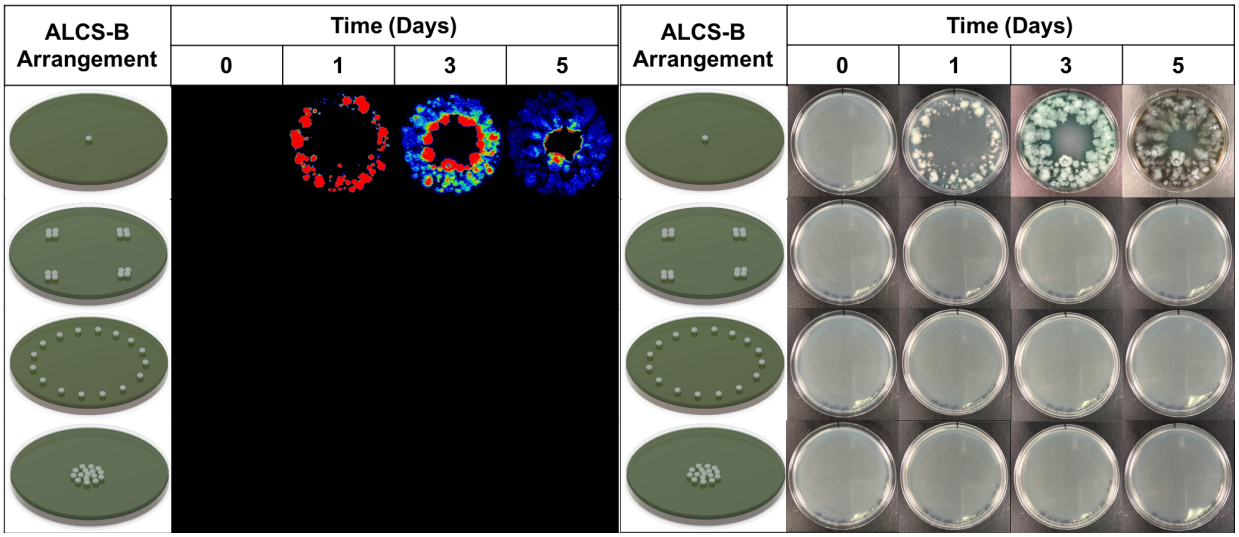

Figure 7A-B. IVIS (A) and white light (B) replica plate images for all of the ALCS-B arrangements that had been previously exposed PA Xen41 biofilm for 7 days. Replica plates were imaged every day for 5 days.

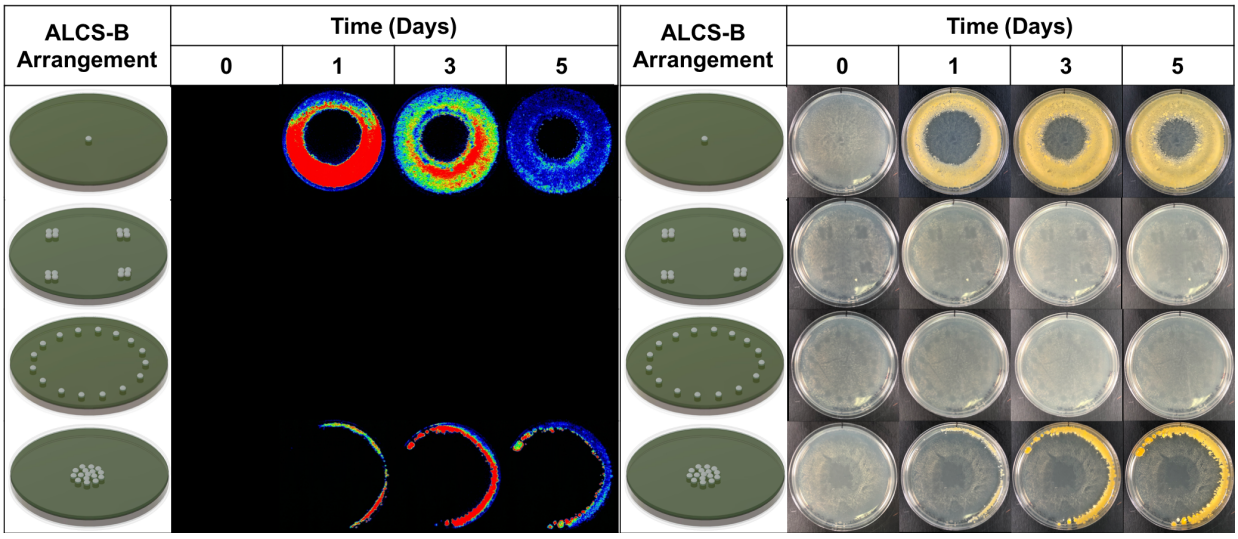

Figure 8A-B. IVIS (A) and white light (B) replica plate images for all of the ALCS-B arrangements that had been previously exposed to SA SAP231 biofilm for 7 days. Replica plates were imaged every day for 5 days.

3.1.3 Zone of Biofilm Inhibition (ZOB-I) vs ALCS-B Arrangement

Zones of biofilm inhibition (ZOB-I) were calculated in terms of area (mm^2) for each ALCS-B arrangement through the first 7 days exposed to PA or SA lawn biofilm. This area of inhibited bacteria was computed using IVIS images and ImageJ image analysis software. The area of the ALCS-B was subtracted from raw ZOB-I measurements, and the corrected areas were then divided by the number of beads to obtain a ZOB-I on a per bead basis. Figure 9A-B shows the ZOB-I (in mm^2 per bead) over time for both PA Xen41 and SA SAP231.

For *P. aeruginosa* Xen41, the sixteen beads arranged circularly around the edge of the plate exhibited the most rapid increase in ZOI, followed by the four clusters of four arrangement. The sixteen beads clustered centrally displayed the slowest inhibition of bacterial activity over time, and did not clear the biofilm until day 4, as mentioned above. In *S. aureus*, the arrangement trend of inhibited bacteria mirrored that of Xen41, though the SAP231 biofilm as a whole prompted a lower rate of inhibition for all arrangements compared to the PA biofilm. This can be visualized by the shallow slopes on Figure 9B compared to the steeper rise of the lines in Figure 9A.

ZOB-I of bead arrangements was also analyzed at the single time point of 1 day after bead placement to further understand the effect of bead clumping on biofilm killing. Figures 10A and 10B compare the number of clumped ALCS-B to the inhibition of PA and SA biofilms, both in terms of ZOB-I and ZOB-I per bead. In both PA Xen41 and SA SAP231, as the number of beads in a cluster rises, the overall area of biofilm cleared also rises; however, the zone of biofilm inhibition per bead falls. In essence, sixteen ALCS-B in a cluster produces more overall biofilm inhibition than four beads in a cluster, but less bacterial inhibition on a per bead basis.

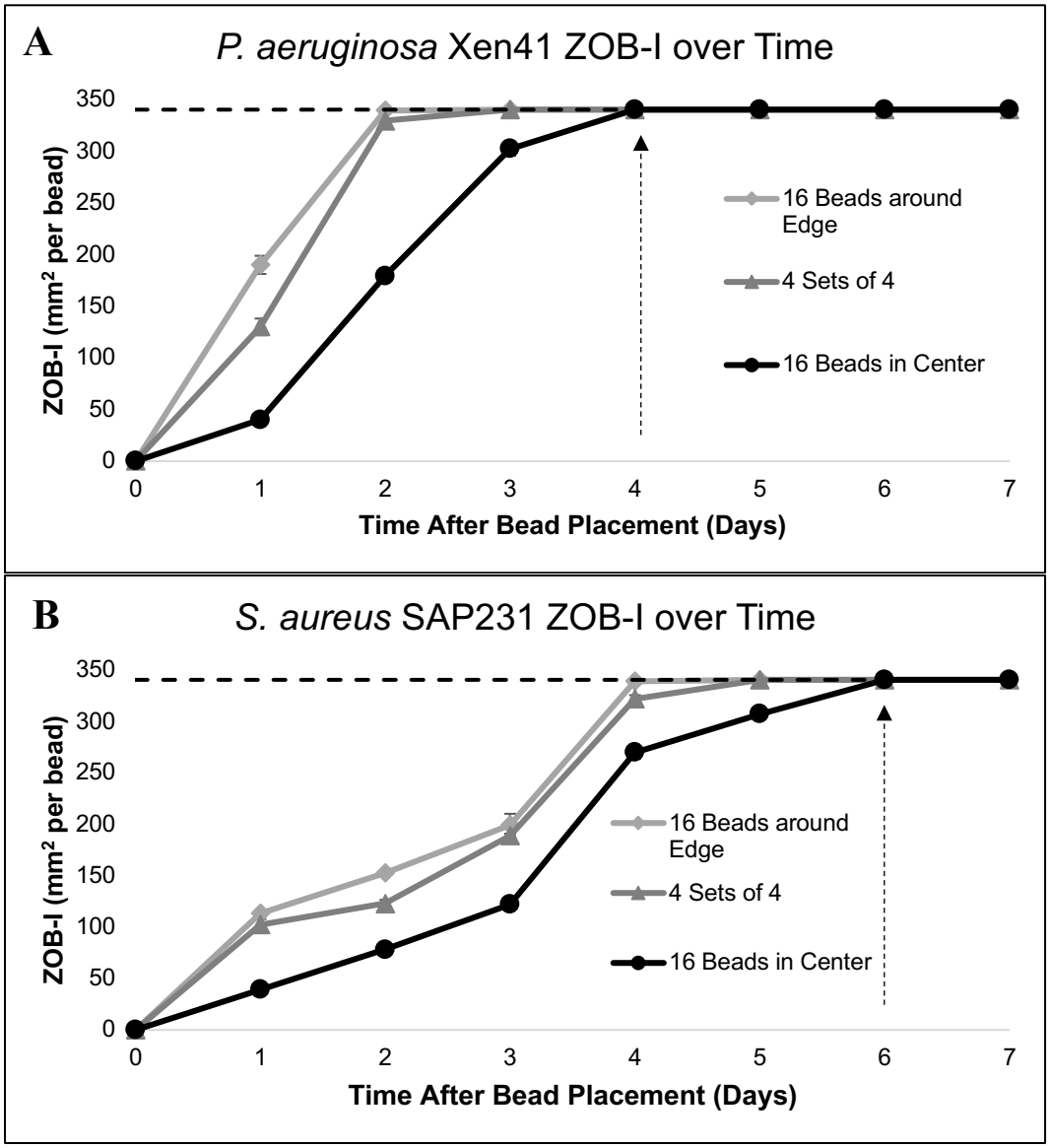

Figure 9A-B. Inhibition of lawn biofilms of *P. aeruginosa* Xen41 (A) and *S. aureus* SAP231 (B) over time, when treated with different VAN + TOB ALCS-B arrangements. Dotted horizontal lines in the graphs represents edges of the Petri dishes and vertical lines represent days of complete lawn biofilm inhibition for all sixteen bead arrangements.

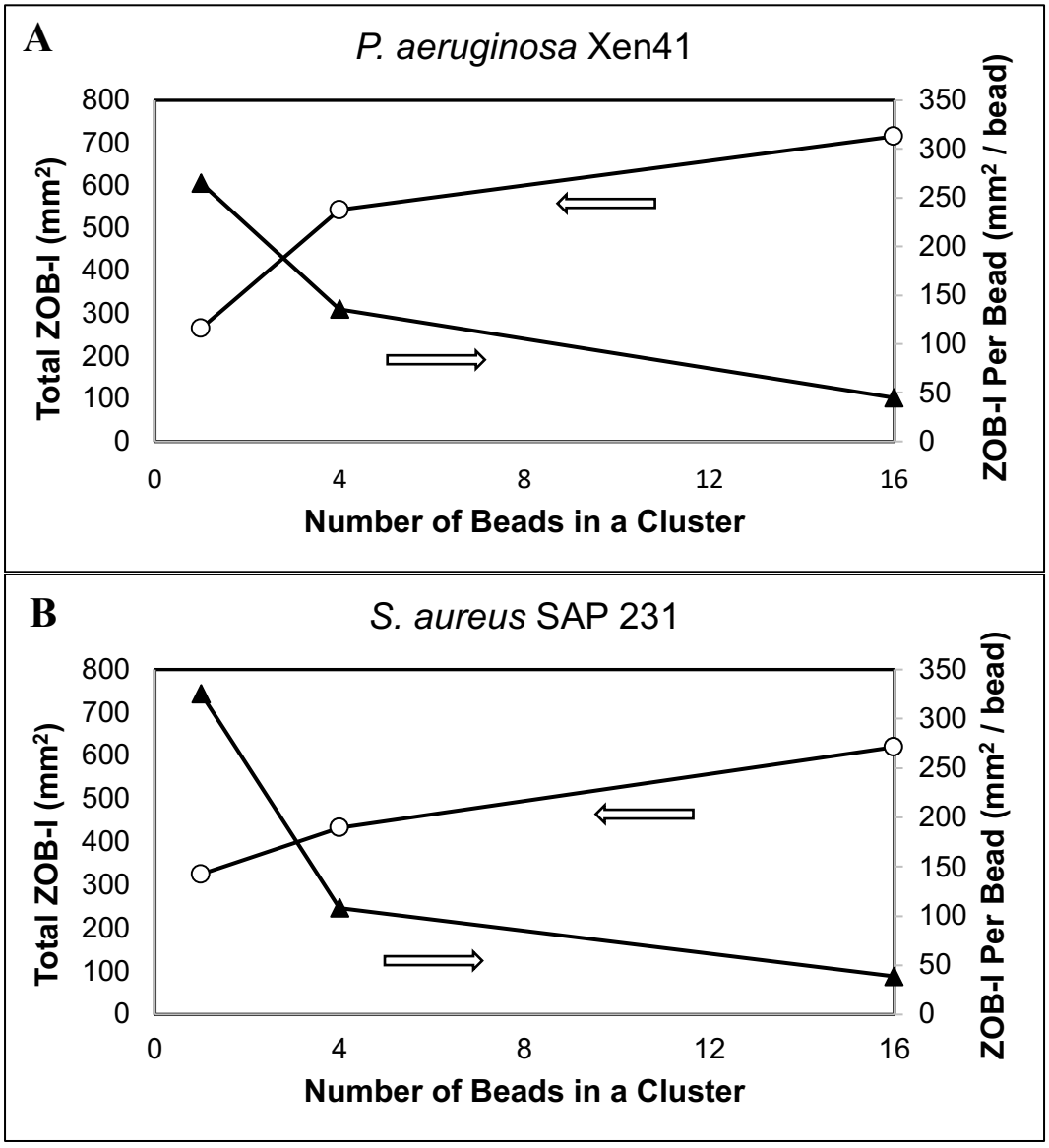

Figure 10A-B. Comparison of zone of biofilm inhibition (ZOB-I) and ZOB-I per bead (both in mm^2) for clusters of 1, 4, and 16 beads. Area of bacteria cleared (ZOB-I) was measured 1 day after ALCS-B were introduced to either P. aeruginosa Xen41 (A) or S. aureus SAP231 (B) lawn biofilm. Arrows on both graphs represent the number axis that refers to the respective data lines. For instance, the triangle data points only refer to ZOB-I per bead (right-hand number axis).

3.2 Large Plate Study

3.2.1 *In Vitro* Imaging System (IVIS) Images Analysis

IVIS imaging was used to visualize the inhibition of *P. aeruginosa* Xen41 biofilm that was introduced to the large plate model on coupons of various materials. As shown in Figure 11B, PA Xen41 biofilm can be visualized under plain light and the effect of ALCS-B can be seen through individual zones of biofilm inhibition (ZOB-I), similar to observations in the bead arrangement study. The large plate with the unloaded spacer (US) exhibited large amounts of bioluminescence over the time period of 5 days. The LS condition also displayed some bacterial activity, though a ZOB-I was generated from the large amounts of VAN+TOB antibiotic in the spacer. However, antibiotic tolerant phenotypes became visible in the LS condition on day 3 (Figure 11A), presenting comparably to the phenotypes seen from the single VAN+TOB ALCS-B placement in the PA Xen41 bead spacing experiment (Figure 5). These tolerant phenotypes were not observed in the LS+LB condition, where bioluminescence was only observed near the edges of the large plate over a period of 5 days.

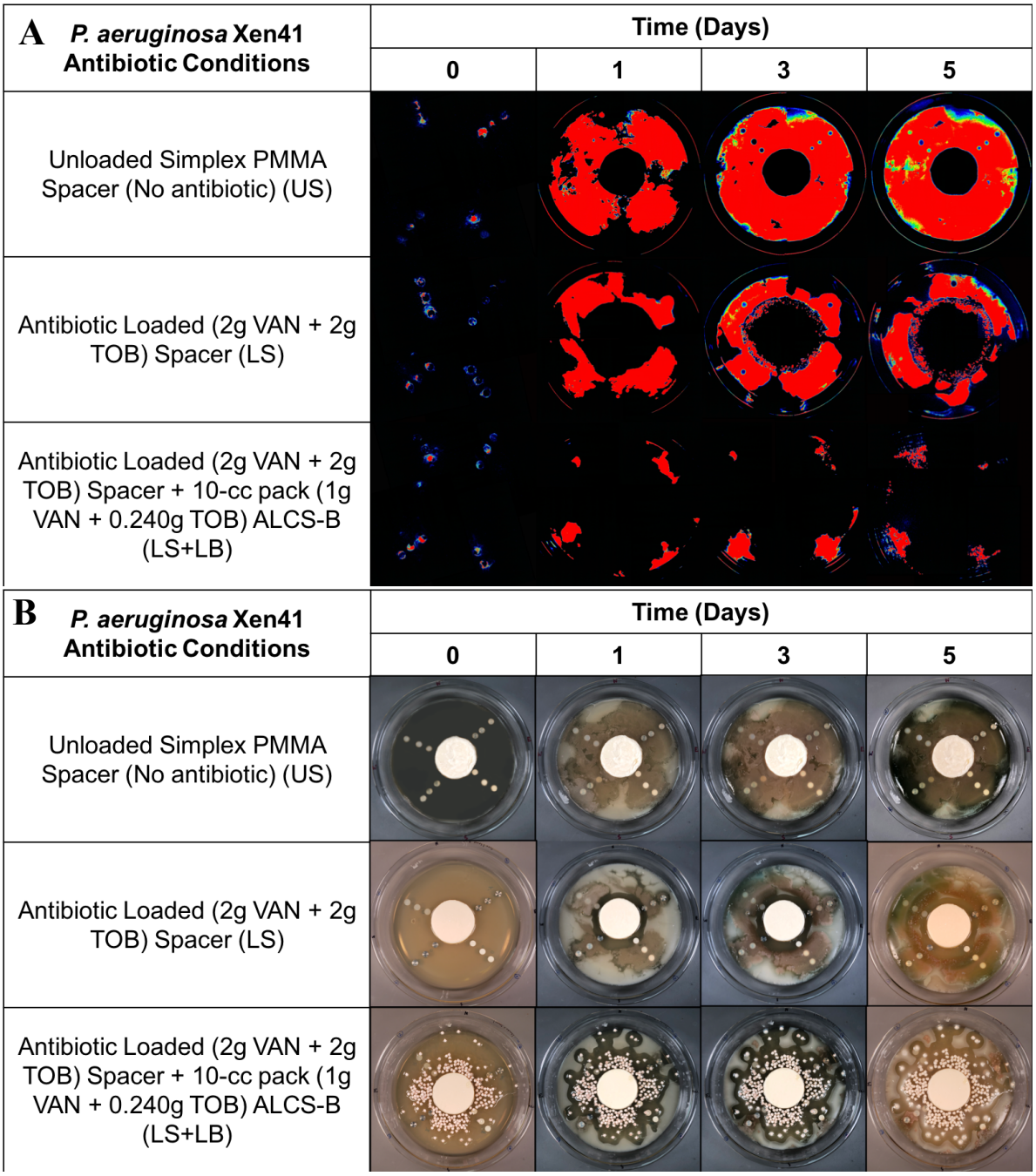

Figure 11A-B. IVIS (A) and white light (B) images tracking the suppression of *P. aeruginosa* Xen41 biofilm activity of three different antibiotic conditions tested by the large plate model. Plates were imaged every 24 hours for 5 days.

3.2.2 Colony Forming Unit (CFU) Counts

CFU counts were performed on coupons initially to confirm bacterial growth, and on day 5 of the large plate model in order to assess bacterial killing. Initial CFU counts of 3-day *P.*

aeruginosa Xen41 on four types of coupons (each in triplicate) exhibited noteworthy bacterial growth, ranging between 8 and 9 log CFU/mL (Figure 12). Polyethylene (PE) coupons exhibited the largest initial CFU count, though it did not significantly differ from any other material.

All coupons for each of the antibiotic conditions were also assessed for number of bacterial cells after 5 days in the large plate model. These counts were conducted in duplicate and shown in Figure 13. The unloaded spacer condition (US), which acted as a no antibiotic control, exhibited sizable CFU counts for all three coupons of each material type. The loaded spacer condition (LS) also showed colonies on all four types of coupons, though growth only occurred on one of three coupons for SS-316 and 2 of 3 coupons for both titanium and hydroxyapatite. Biofilm was present on all three polyethylene coupons, though the CFU counts were low for PE coupon #1, increased for PE coupon #2 and amplified even more for PE coupon #3. Hence, the increased bacterial colonies on PE coupons in the LS condition correlates positively with the distance of the coupon from the spacer.

The LS+LB condition exhibited CFU counts for both PE and HA coupons, but no colonies were present for SS-316 and Ti coupons. Further, bacterial growth was seen only on HA coupon #3, PE coupon #2, and PE coupon #3. The log CFU/mL for HA coupon #3 did not differ significantly from HA coupon #3 in either the US or LS conditions, while the CFU counts all other coupons in the LS+LB condition, including coupons PE #2 and PE #3, were significantly different compared to the same coupons for the US and LS antibiotic circumstances. For instance, LS+LB PE coupon #2 displayed a log CFU/mL of 2.42, while the LS condition PE #2 had a count of 7.20 log CFU/mL, and the US condition possessed a count of 8.98 log CFU/mL.

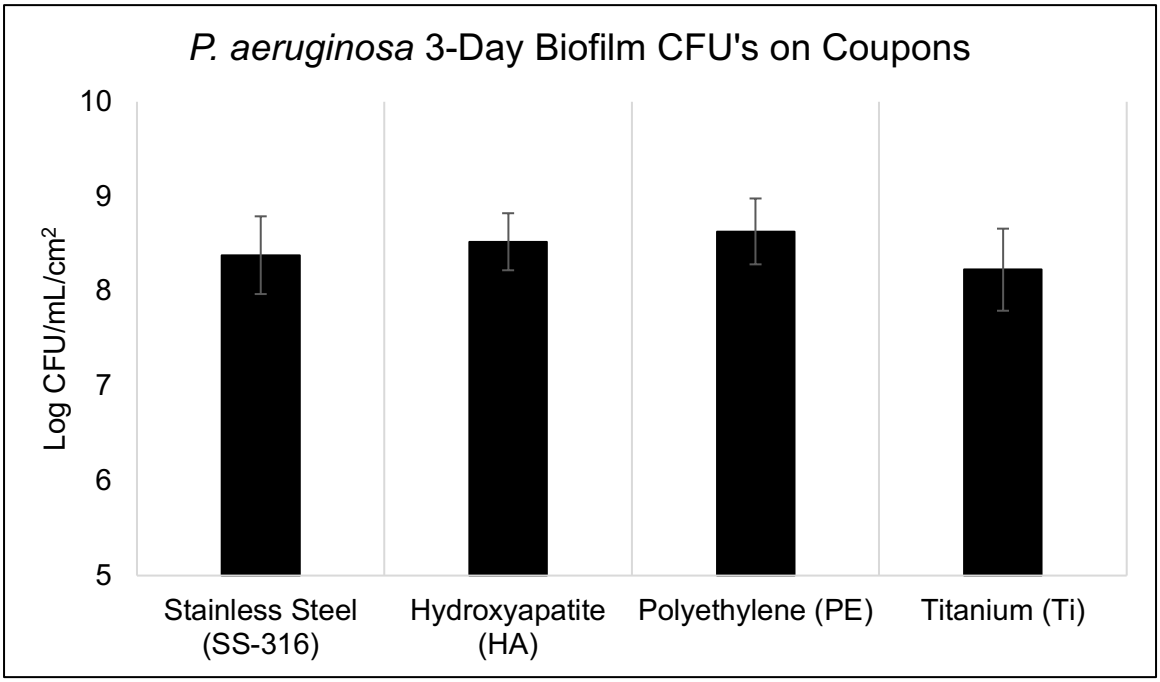

Figure 12. CFU counts (in $Log_{10}CFU/mL/cm^2$) of *P. aeruginosa* Xen41 3-day biofilm on coupons of four materials common to orthopedic prosthetic implants.

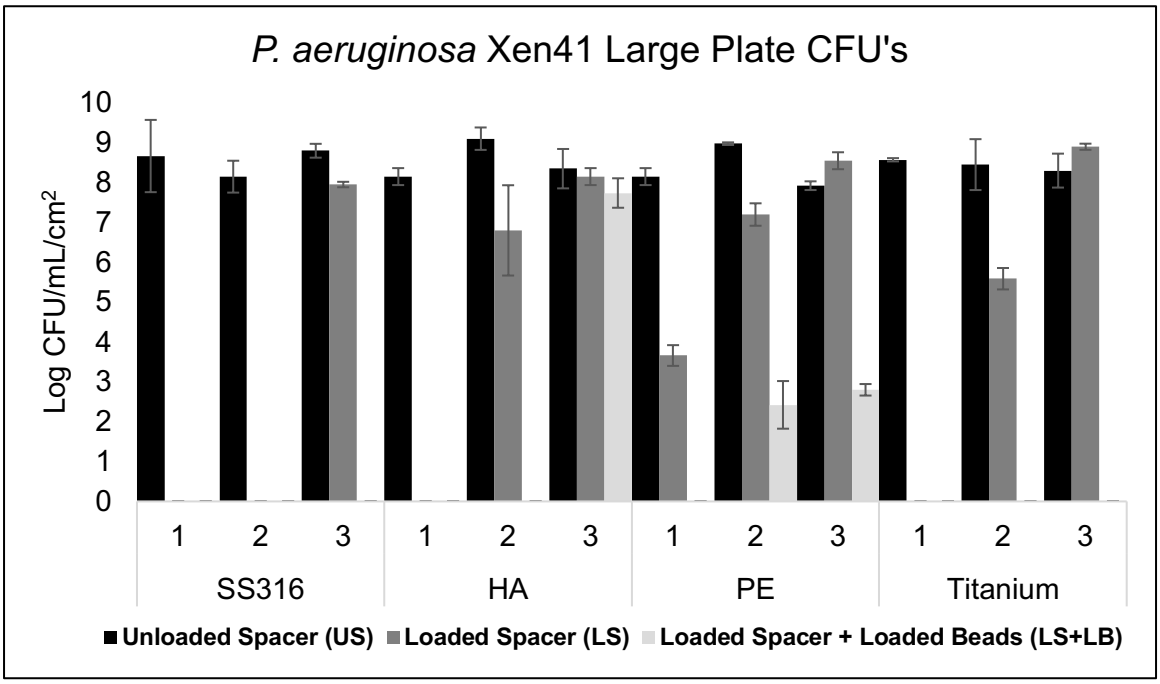

Figure 13. CFU counts of *P. aeruginosa* Xen41 biofilms after being grown for 3 days in TSB media and then introduced into the big plate model against one of three antibiotic conditions for 5 days. Coupons are labeled 1, 2, and 3, where coupon #1 denotes the coupon closest to the PMMA spacer, coupon #2 represents the intermediate coupon, and coupon #3 signifies the coupon closest to the plate's edge.

4. Discussion

4.1 The Importance of ALCS-B Spacing on Biofilm Eradication

The effect of ALCS-B spacing on biofilm eradication was studied due to its low amount of understanding when using these materials to treat infected arthroplasties. The importance of bead spacing was visualized through analyzing the zone of biofilm inhibition (ZOB-I) for each $CaSO_4$ bead arrangement, while also using the bioluminescence of PA Xen41 and SA SAP231 to visually track this suppression of bacterial metabolic activity. From the IVIS images of both pathogens, it was observed that arrangements containing large clusters of beads require more time to eradicate the biofilm compared to ALCS-B arrangements that are spaced out. The sixteen-bead cluster in the center of the plate, for example, cleared both *P. aeruginosa* Xen41 and *S. aureus* SAP231 lawn biofilms one day later than the other two sixteen-bead arrangements, based on IVIS images (Figures 5 and 6).

Nevertheless, all three sixteen-bead arrangements did fully inhibit both Xen41 and SAP231 lawn biofilms over the time period of 7 days compared to one ALCS-B, which suggests that high concentration of antibiotic infused in ALCS-B can completely inhibit a bacterial lawn over a time of 7 days. Moreover, all sixteen-bead arrangements produced no antibiotic tolerant phenotypes, unlike the single bead placements in Xen41. This suggests that antibiotic tolerance can perhaps be avoided by either large number of ALCS-B or by increased concentration of antibiotics.

Still, IVIS imaging over 7 days does not confirm full eradication of biofilms, and replica plating was used to distinguish between inhibited and killed bacteria. In essence, initial IVIS imaging that suggests full bacterial killing could be shown as bacterial inhibition by growth on a fresh agar plate. However, the lack of growth on replica plates of all sixteen-bead arrangements

for PA Xen41 (Figure 7) does indicate that all biofilm had been eradicated by the antibiotic treatment. In *S. aureus* SAP231, the four clusters of four bead arrangement and the circular edge replica plates also confirmed total biofilm eradication. The sixteen-bead central cluster, however, demonstrated growth when replica plated (Figure 8), suggesting that only the middle portion of the *S. aureus* lawn had been eradicated, and the edges had not been fully killed by antibiotic. This also strengthens the notion that spacing of antibiotic loaded $CaSO_4$ beads is important in treating lawn biofilm infections, since the same concentration of antibiotic in a clustered arrangement did not fully kill a *S. aureus* biofilm, while other, more spaced arrangements did eradicate the same lawn.

This importance of ALCS-B spacing can be applied to surgeons' treatment protocol periprosthetic joint infections. In treating these biofilm infections with ALCS-B, surgeons many times have no set etiquette on how to space the beads. Moreover, there is little knowledge on if such a procedure is necessary. The results from testing different ALCS-B arrangements in vitro suggest that spacing of ALCS-B is an important consideration in order to 1) fully eradicate biofilm infections, and 2) kill the bacteria in a timely manner.

Tracking the zone of biofilm inhibition (ZOB-I) over time was used to determine the importance of ALCS-B spacing in doing the latter. Through analyzing the rate of biofilm inhibition, the data proposed that arrangements with more spread out ALCS-B killed all bacteria on the plate more quickly than clustered bead arrangements (Figure 9). This was observed for both *P. aeruginosa* and *S. aureus* biofilm and suggests that a more evenly spaced out implementation of ALCS-B may be most efficient in inhibiting these lawns that present in many periprosthetic joint infections (PJIs).

With this in mind, ZOB-I data was manipulated to better display the adverse effect of bead clustering (Figure 10). As the number of ALCS-B that were clustered increased, the overall area of inhibited bacteria increased. This is consistent with the idea discussed above that an increased number of beads containing antibiotic and a larger concentration of antibiotic will increase the zone of biofilm inhibition. Yet, the same increase in beads clustered together also prompted a decrease in ZOB-I per bead. This approach, which corrects for the increased concentration of antibiotic, depicts the true downward trend in bacterial inhibition that results from increased clustering. This trend, observed for both *P. aeruginosa* and *S. aureus*, suggests that avoiding clustered bead placement when treating periprosthetic joint infection may allow for increased bacterial inhibition and a more efficacious clearance of the infection.

The study of ALCS-B arrangement against *P. aeruginosa* and *S. aureus* lawn biofilms yielded data confirming the importance of bead spacing in efficiently eliminating these types of infections in vitro. Moreover, the study indicated that in vitro lawn biofilms of bioluminescent Xen41 and SAP231 bacterial strains could be fully eradicated through the use of sixteen VAN+TOB antibiotic-loaded $CaSO_4$ beads, verifying the significance of ALCS-B in treating infections of *P. aeruginosa* or *S. aureus* that can present clinically following total joint arthroplasties.

4.2 The Extended Biofilm Killing of ALCS-B Beyond the Limits of Spacer

The influence of ALCS-B in conjunction with the presence of PMMA spacers was also evaluated through a large plate model with *P. aeruginosa* Xen41 biofilm grown on four types of coupons. In vitro bioluminescence imaging (BLI) showed the inhibition of biofilm over the course of 5 days, similar to the ALCS-B spacing study. In the first antibiotic condition, which

contained only an unloaded spacer with no antibiotic (US), biofilm was seen covering the entirety of the large plate over all 5 days (Figure 11). This condition, which acted as a negative control, demonstrates the result of no antibiotic action on a *P. aeruginosa* periprosthetic joint infection (PJI) and suggests the rapid growth of biofilm once contamination is introduced to a prosthetic during a total joint arthroplasty.

Similarly, the second condition containing only a loaded spacer (LS), illustrated the effect of solely implementing a PMMA spacer infused with 2 grams of both VAN and TOB antibiotic, while the third condition also included a 10-cc pack of VAN+TOB ALCS-B (LS+LB). Though the LS condition showed slightly less bioluminescence compared to the US control, the LS+LB condition seemed to outperform the LS condition over 5 days (Figure 11). The imaging suggests that the presence of ALCS-B in combination with an antibiotic loaded spacer can produce much more inhibition of biofilm, though the concentration of antibiotic in the beads is much less than in the loaded spacer. Since the loaded PMMA spacer contained 2 grams of both Vancomycin and Tobramycin (compared to the 1 g of VAN and 0.240 g TOB in the 10-cc pack ALCS-B), it would be expected that the loaded spacer would be more essential in diminishing bacterial activity than antibiotic loaded $CaSO_4$ beads. Yet, as first observed by imaging, this does not appear accurate. The LS condition and the US condition appeared to produce similar bioluminescent levels and inhibition of *P. aeruginosa* Xen41 biofilm, while the implementation of ALCS-B produced further bacterial suppression. This displays the strong positive effect of the ALCS-B in clearing these infections when used in combination with loaded PMMA spacers, even with the lower concentrations of antibiotic.

CFU counts confirmed this trend visualized with IVIS imaging. After verifying initial 3-day biofilm growth on four different types of coupons (Figure 12), CFU counts were also taken 5

days after these coupons were employed into the large plate model (Figure 13) to examine the difference in bacterial growth between the three antibiotic conditions. The LS+LB condition produced significantly less growth than both the LS and US conditions on all coupons, aside from hydroxyapatite (HA) coupon #3. Moreover, the LS+LB condition produced no growth on nine of the twelve coupons in the large plate model, while the LS condition showed no growth on four coupons (Figure 13). This aligns with the IVIS imaging, and once again shows the increased importance of ALCS-B in clearing in vitro *P. aeruginosa* biofilm, which can be applied to biofilm infection in joint replacement surgeries.

This effect could be due to the decreased mechanical strength of $CaSO_4$ compared to Poly(methyl) methacrylate, which might allow antibiotic to elute more favorably than in the strong fabric of PMMA. As surgeons commonly use PMMA spacers to treat PJIs because of their ability to both contain antibiotic and help stabilize the implant, ALCS-B are added less frequently. However, the large plate study suggests the increased importance of these loaded beads in providing a more widespread antibiotic treatment with the ability for increased bacterial clearance when compared with a PMMA spacer.

In addition, the implementation of biofilm onto coupons was meant to simulate periprosthetic joint infection by allowing *P. aeruginosa* to adhere to common materials used in prosthetic joint implants. Coupon of hydroxyapatite and polyethylene generated more bacterial growth rather than SS-316 and Ti (Figure 13), which could be explained by the rough and uneven nature of these materials compared to the smooth surface of SS-316 and Ti coupons. This would allow bacteria to stick in crevices of the slightly more jagged material, causing the biofilm to be more evasive to common antibiotic treatment. Nevertheless, the large plate model allowed

biofilm to be grown on relevant orthopedic material to provide a relevant challenge for PMMA and $CaSO_4$ antibiotic treatment.

The large plate study showed the significant importance of ALCS-B in treating in vitro P. *aeruginosa* Xen41 biofilm, which can be related to infections in the clinic. Though surgeons look to PMMA spacers with large amount of antibiotic over $CaSO_4$ beads in treating these infections, the large plate study suggests that ALCS-B have a larger role in eradicating biofilm beyond the limits of antibiotic infused PMMA spacers due to their ability to provide widespread coverage and sustained release of antibiotic. It could be true that the compact and dense puck shape of the PMMA spacer used in the study did not fully allow for its true antibiotic elution to occur, since surgeons commonly shape this PMMA material into many forms to appropriately suit the orthopedic implant; however, this could merely be an explanation for the less efficacious antibiotic elution of the LS condition obtained in the study compared to the LS+LB condition. Nonetheless, the large plate study demonstrated the increased efficacy of ALCS-B in inhibiting *P. aeruginosa* biofilm, which, in conjunction with the previous bead spacing experiment, can be used to call for added use of these antibiotic loaded $CaSO_4$ materials in operating rooms. Though not aiding structurally, $CaSO_4$ offers vital antibiotic release advantages that provide significant and efficacious killing of in vitro biofilm when used in addition to standard antibiotic loaded PMMA spacers.

5. Conclusions and Future Works

Due to the morbidity and mortality resulting from periprosthetic joint infection, examining multiple methods and means of antibiotic treatment is crucial in the treatment of patients with these infections. By further understanding the importance of antibiotic loaded calcium sulfate bead spacing along with the positive effect of ALCS-B implementation in combination with antibiotic loaded PMMA spacers, antibiotic treatment methods are able to grow towards defeating biofilm infections associated with orthopedic surgery.

Antibiotic loaded $CaSO_4$ beads were shown as an encouraging and advantageous mechanism in treating periprosthetic joint biofilm infections and preventing further infection after revision to infected arthroplasties, suggested based on the application of in vitro studies. In treating these infections, spacing of ALCS-B was determined an important consideration in order to more efficaciously inhibit and eradicate *P. aeruginosa* and *S. aureus* lawn biofilm. Moreover, when applied more uniformly to a lawn compared to clumped together, total biofilm eradication was achieved. ALCS-B were shown to effectively eliminate lawn biofilms and any antibiotic tolerance when used in sufficient number, antibiotic concentration, and spacing arrangement, however, low number of ALCS-B and clustered spacing resulted in antibiotic tolerance and inadequate biofilm killing, respectively. In the future, further study of Stimulan ALCS-B spacing is intended to find the most effective pattern for eradicating bacterial biofilm infections. This will be done by testing perfectly equidistant ALCS-B arrangements, as well as narrowing in on the number of beads needed to fully eradicate an in vitro lawn biofilm. The objective is to determine a specific and effective protocol to completely eradicate these bacteria in vitro, which can then be related to, studied, and eventually implemented in clinical settings.

The large plate model was also used to suggest significance of these beads in addition to the antibiotic loaded PMMA spacers, which are more commonly used in operating rooms. In vitro antibiotic conditions of an antibiotic loaded PMMA spacer with a 10-cc pack of Stimulan ALCS-B produced significantly less growth of *P. aeruginosa* biofilm on polyethylene, titanium, stainless steel, and hydroxyapatite coupons compared to conditions with only an antibiotic loaded spacer. Moreover, use of ALCS-B in addition to a loaded spacer resulted in a lack of the antibiotic tolerant phenotypes observed in the loaded spacer condition. Visual and quantitative measures confirmed this increased usefulness of ALCS-B in inhibiting *Pseudomonas* biofilm. When applied to clinical scenarios, these beads are shown to perhaps offer important and additional value in treating biofilm infections resulting from total joint arthroplasties.

Our lab group hopes to continue utilizing a refined large plate model to test the importance of antibiotic treatment on eradicating biofilm infection. Different pathogens such as *S. aureus* will be tested in the model under mannitol salt agar to minimize contamination. Additionally, biofilm will be implemented into the agar instead of on coupons to avoid ambiguous spreading of bacteria and allow a more uniform lawn to result. Different ALCS-B spacings will also be employed into the large plate model so that aspects of the bead spacing study can be employed into an area that is not limited to the small area of a petri dish. Thus, future work using this model will continue to analyze and refine relevant antibiotic treatment methods against in vitro biofilms meant to simulate orthopedic periprosthetic joint infection.

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Supplementary Figures

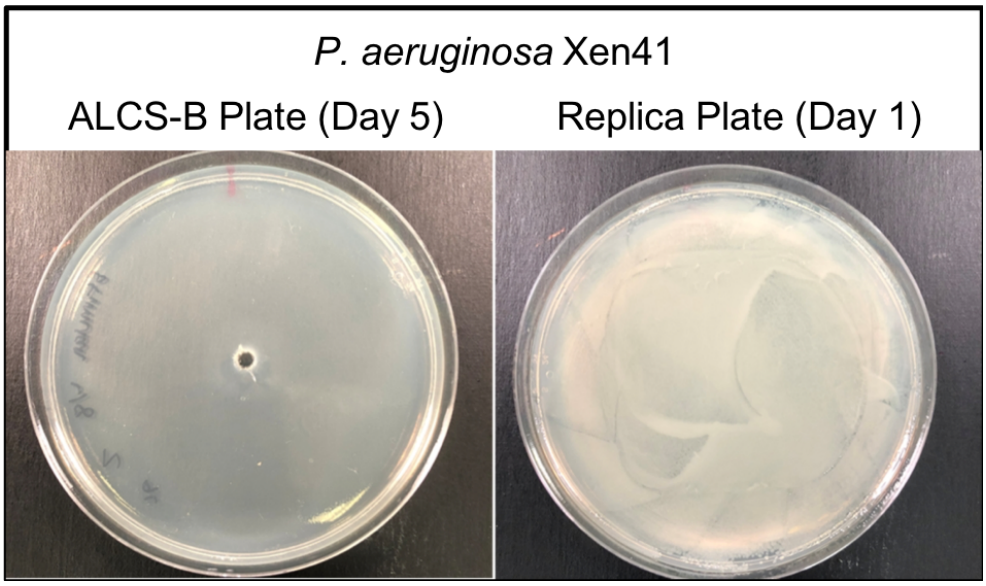

Figure S1. Images of antibiotic carryover during replica plating. One Tobramycin Stimulan ALCS-B was placed on 24-hour *P. aeruginosa* Xen41 lawn biofilm, incubated at 37 ˚C for 5 days, and then replica plated onto a fresh TSA agar plate. A *P. aeruginosa* Xen41 culture was then spread on the replica plate. No antibiotic inhibition of PA Xen41 growth was observed (shown on the right), indicating that there was no residual antibiotic after replica plating.

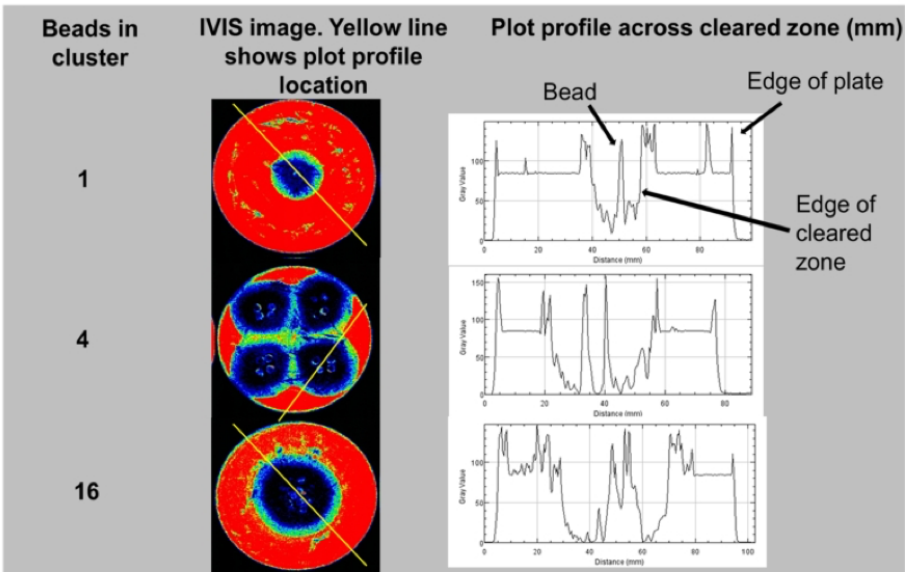

Figure S2. ImageJ image analysis of ALCS-B arrangement plates to generate ZOB-I measurements shown in Figure 10. Plot profiles of 1, 4, and 16 clustered beads were generated using ImageJ. Spikes in the plot profiles depict the edge of the beads, edge of the bacterial zone of clearance, and edge of the plate, so that corrected bacterial clearance measurements could be obtained (in mm), and then converted to area (mm^2) using the area equation of a circle.